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A CELLULAR STUDY OF TEOSINTE *ZEA MAYS* SUBSP. *PARVIGLUMIS* (POACEAE) CARYOPSIS DEVELOPMENT SHOWING SEVERAL PROCESSES CONSERVED IN MAIZE[1]

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The evolutionary history of maize (*Zea mays* subsp. *mays*) is of general interest because of its economic and scientific importance. Here we show that many cellular traits described previously in developing caryopses of maize are also seen in its wild progenitor teosinte (*Zea mays* subsp. *parviglumis*). These features, each with a possible role in development, include (1) an early programmed cell death in the maternal placento-chalazal (P-C) layer that may lead to increased hydrolytic conductance to the developing seed; (2) accumulation of phenolics and flavonoids in the P-C layer that may be related to antimicrobial activity; (3) formation of wall ingrowths in the basal endosperm transfer layer (BETL); (4) localization of cell wall invertase in the BETL, which is attributed to the increased transport capacity of photosynthates to the sink; and (5) endoreduplication in endosperm nuclei suggested to contribute to increased gene expression and greater sink capacity of the developing seed. In maize caryopsis, these cellular traits have been previously attributed to domestication and selection for larger seed size and vigor. Given the conservation of the entire cellular program in developing teosinte caryopses described here, we suggest that these traits evolved independently of domestication and predate human selection pressure.

Key words: cell wall invertase; endoreduplication; genome size; maize; Poaceae; programmed cell death; teosinte; *Zea mays* subsp. *parviglumis*.

The domestication of all major crop plants, including maize (*Zea mays* subsp. *mays*), occurred during a brief period in human history between 5000 and 10 000 years ago. Microsatellite-based phylogeny of maize shows all maize to be in a single monophyletic lineage that is derived from a wild grass native to Mexico and Central America—teosinte (*Zea mays* subsp. *parviglumis*) (Matsuoka et al., 2002). This analysis and others support a single domestication for maize and demonstrate *Zea mays* subsp. *parviglumis* to be its sole progenitor (Matsuoka et al., 2002). However, maize and teosinte have such extreme differences in their adult morphologies that taxonomists initially considered teosinte to be more closely related to rice than to maize. The most dramatic distinctions between teosinte and maize concern the architecture of their female inflorescences or ears and the structure of their caryopses (Fig. 1) (Doebley et al., 1990, 2004). Specifically, the maize ear is nondisarticulating at maturity (Doebley et al., 1990), presumably from the lack of an abscission layer. In addition, the maize caryopsis is uncovered on the surface of the ear, while the teosinte caryopsis is tightly encased in structures called cupulate fruitcases (Fig. 1). Recent molecular quantitative trait locus (QTL) mapping has successfully described the genetic background leading to the unbranched plant architecture of maize (studies of the gene *teosinte branched 1*, *tb1*) (Doebley, 2004; Wang et al., 2005; Clark et al., 2006) and the liberation of the maize caryopsis from the hardened cupulate fruitcase of teosinte (studies of the gene *teosinte glume architecture*, *tga 1*) (Doebley 2004; Wang et al., 2005). Additionally, as shown by association mapping, major regulatory genes, e.g., maize homologs of *FLORICAULA*, *zfl2* and *APETALA1*, *zap1*, have a role in the natural variation of complex traits in teosinte (Weber et al., 2007), and it has been suggested that genes such as *ramose* genes *ra1* and *ra2* are associated with ear structure and that the MADS-box gene, *zagl1* is associated with ear shattering (Weber et al., 2008). In contrast with many genetic studies, cellular level studies on caryopsis development in teosinte have not been thoroughly investigated. It is not known whether these mechanisms are similar to those in maize or to what extent they may contribute to the overall change in the maize caryopsis phenotype. To resolve these questions, we need to integrate our knowledge from several biological disciplines, including genetics, cytology, and anatomy.

The caryopsis, the single-seeded fruit of plants from the grass family, is composed of three major parts: the pericarp, embryo, and endosperm. The pericarp is derived from the ovary wall and adheres strongly to the seed coat formed from the integuments of the ovule. At fertilization, the ovule consists of the embryo sac, enclosed by the nucellus and integuments. The products of double fertilization are a diploid zygote and a triploid primary endosperm cell. The latter develops into a storage endosperm tissue that is structurally adapted to ensure efficient translocation of nutrients to the developing embryo. Cereal endosperm undergoes three distinct stages: syncytial, mitotic, and differentiation (Olsen, 2004; Nguyen et al., 2007). Following fertilization, the primary endosperm nucleus passes through multiple rounds of division without cytokinesis, resulting in a syncytium or multinucleate cytoplasm. Cell walls then form around individual nuclei so that the endosperm becomes multicellular.

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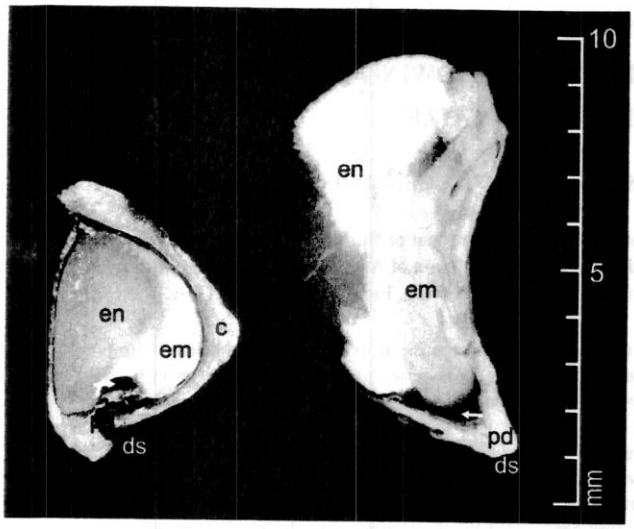


Fig. 1. Photograph of longitudinal sections of mature caryopses of teosinte (*Zea mays* subsp. *parviglumis*, left) and maize (*Z. mays* subsp. *mays* inbreed line W22, right). The arrows point to the placento-chalazal layer, which includes the black layer. c, cupulate fruitcase; ds, disarticulation site; em, embryo; en, endosperm; pd, pedicel.

A series of mitotic cell cycles follows, establishing the basic cell number in the body of the endosperm. The next developmental stage includes endoreduplication. The process is not associated with an increasing cell number, but involves continued DNA replication without subsequent mitosis, leading to endopolyploid nuclei with polytene chromosomes (Joubès and Chevalier, 2000). Endoreduplication occurs soon after cell divisions, before the onset of the storage phase, and has been seen in all cereal endosperms studied thus far (Vilhar et al., 2002; Kladnik et al., 2006).

The basal endosperm transfer cell layer (BETL) at the base of the endosperm provides the primary interface between maternal and filial tissues in developing maize caryopsis (reviewed in Royo et al., 2007). The BETL cells are readily identifiable 7–9 d after pollination (dap) (Kiesselbach, 1949; Schel et al., 1984) by their distinctive cellular morphology, that is well developed at 16 dap, when the maize BETL is 65–70 rows of cells wide and 3–6 cells deep (Davis et al., 1990). The transfer cells have been characterized as having extensive finger-like wall ingrowths that protrude into the cytoplasm and amplify the surface area of the plasma membrane enriched in nutrient transporters (Davis et al., 1990; Offler et al., 2003). Thus, transfer cells are thought to be actively involved in intensive, short-distance, intercellular transportation of photosynthates. Several lines of new evidence suggest that a normal BETL with uniform wall ingrowths is essential to proper seed development. Notably, Maitz et al. (2000) reported a *reduced grain filling* (*rgf1*) locus that greatly reduces expression of maize BETL markers, with a loss of 70% seed mass at maturity. Similarly, *globby1* (Costa et al., 2003), *empty pericarp4* (Gutierrez-Marcos et al., 2007), and *baseless1* (Gutierrez-Marcos et al., 2006) maize mutants have an abnormal BETL at an early stage of seed development and, ultimately, lethal, aborted seed phenotypes. Although transcriptome analyses show 5504 endosperm-specific expressed sequence tags (ESTs) (Lai et al., 2004), only a small number of genes are thus far known to be directly involved in the transport processes (Chourey et al., 2006; Royo et al., 2007). Transport adaptations were thus assumed to be determined by the regulation of the timing of expression, location, and activity of the gene products. In addition to genes associated with transport, several transfer-cell-specific genes encoding small, extracellular, hydrophilic proteins have also been identified. Although a functional role for these proteins is not yet known, indirect evidence supports their involvement in defense against pathogens (Royo et al., 2007).

The major structural bridge in the transfer of photosynthates and nutrients from the mother plant to a developing caryopsis is the pedicel. Placento-chalazal (P-C) cells within the pedicel are positioned immediately below the basal endosperm cells and are believed to have a critical role in the postvascular transport of water, sugars, and nutrients. The maize P-C layer is also presumed to function in the antimicrobial protection of the developing seed through the accumulation of phenolic compounds (Kladnik et al., 2004; LeClere et al., 2007), with antifungal properties (Serna et al., 2001; Cai et al., 2002).

An additional feature during the development of the P-C layer in maize is programmed cell death (PCD). PCD in the P-C layer shares many cellular level similarities with the formation of xylem, a major water transport system in plants (Kladnik et al., 2004, 2005). PCD is a physiological process that leads to selective elimination of either unwanted cells in animals called apoptosis or systematic degradation of all nuclear and cytoplasmic contents in plants. In maize, the development of the P-C layer includes two distinctive cell death programs; one is autolytic, and the second is more apoptosis-like (Kladnik et al., 2004, 2005).

In the work reported here, we show that the various cellular processes in developing caryopses of maize (Vilhar et al., 2002; Kladnik et al., 2004; Cheng et al., 1996), which are attributed to the selection for larger seed size, are to a great extent evolutionarily conserved because they also occur in the development of teosinte caryopsis. These processes include endoreduplication, the development of transfer cells in the BETL, and the development and differentiation of the P-C layer through PCD.

MATERIALS AND METHODS

Plant material—Balsas teosinte (*Zea mays* L. subsp. *parviglumis* H. H. Iltis, J. F. Doebley & R. Guzman) seeds (accession 21785 and 21812) are from the U. S. National Plant Germplasm System, Iowa State University, Regional Plant Introduction Station, Ames, Iowa. Seeds originated from Guererro, Mexico. In 2008, the seeds of teosinte were grown to maturity in greenhouses in Slovenia (46°03′N, 14°28′E) and in Florida, USA (29°38′N, 82°21′W). Flowers were open-pollinated in the middle of December in Slovenia and at the beginning of October in Florida. Only caryopses of the line 21785 were histologically examined in detail.

Seeds of maize (*Zea mays* L. subsp. *mays*, inbred line W22) were grown to maturity in a greenhouse in Florida, USA (29°38′N, 82°21′W) during different growing seasons without visible changes in caryopsis development (Vilhar et al., 2002; Kladnik et al., 2004, 2005; LeClere et al., 2007; Chourey et al., 2006).

Caryopses of teosinte were collected on different days after pollination (dap) at the Slovenia and USA locations, and all traits were measured on both groups to check for any developmental alterations that may have been due to different latitudes.

Seeds of maize (*Zea mays* subsp. *mays*, inbred lines W22 and CE-777) were used for the measurement of nuclear DNA content.

The seed vouchers were deposited in Herbarium LJU.

Measurement of nuclear genome size—Caryopses of teosinte and maize were surface-sterilized for 15 min in 1.5% sodium hypochlorite solution, rinsed with tap water, left to imbibe overnight, then germinated in sprouting trays. Teosinte caryopses were soaked in 1% hydrogen peroxide overnight to induce germination (Naredo et al., 1998). Root tips were then processed according to

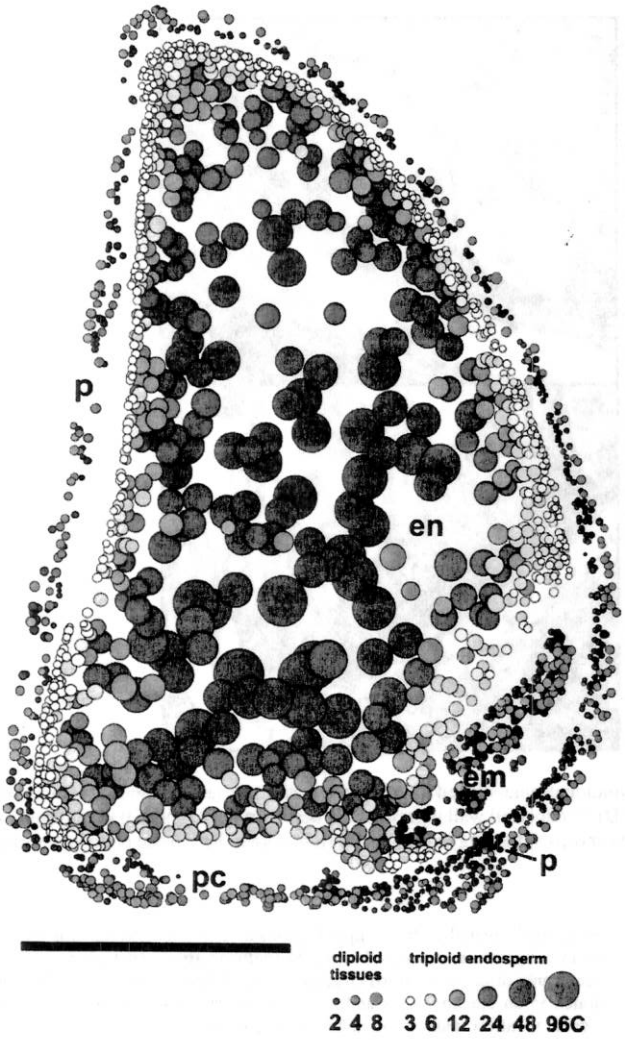


Fig. 2. Spatial distribution of endoreduplication in teosinte caryopsis. In situ DNA content of nuclei is shown in the median longitudinal tissue section of teosinte caryopsis 11 d after pollination. Nuclei of different endopolyploidy classes are color-coded; diameter of bubbles is linearly related to the diameter of measured nuclei. em, embryo; en, endosperm; p, pericarp; pc, placento-chalazal layer. Bar = 1 mm.

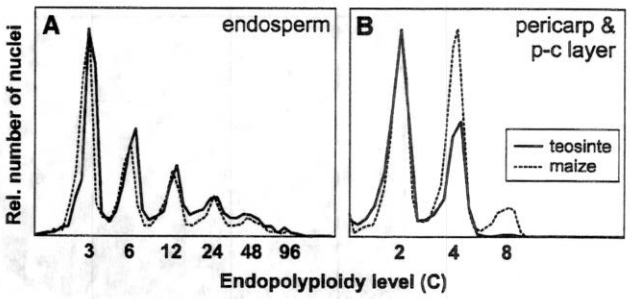


Fig. 3. Frequency distribution of relative number of nuclei with different nuclear DNA amounts in caryopses of teosinte and maize. (A) Endosperm and (B) maternal tissues (pericarp and placenta-chalazal layer) in representative median longitudinal sections of teosinte 11 d after pollination (dap) (1119 nuclei in endosperm and 841 nuclei in maternal tissues) and maize 12 dap (1825 nuclei in endosperm and 3851 nuclei in maternal tissues). Frequency distributions are normalized to the highest peak values.

Dolenc Koce et al. (2003). Root tips (ca. 1 cm long) excised from the germinated caryopses were fixed in 4% formaldehyde and stained with Feulgen reagent according to the protocol in Dolenc Koce et al. (2003). The Feulgen reaction is quantitative for DNA if the only aldehydes remaining in the cell are those produced from the hydrolysis of DNA (Feulgen and Rossenbeck, 1924). Squash preparations of dissected apical root meristems were prepared in 45% acetic acid, frozen on dry ice, then rinsed in 96% ethanol. The amount of nuclear DNA was measured by DNA image cytometry, using the interphase-peak method developed in our laboratory (Vilhar et al., 2001) and expressed as a 2C-value (1C represents the nuclear DNA content of a nonreplicated haploid genome). The image analysis instrumentation was as described in Bačič et al. (2007) and calibrated according to Vilhar and Dermastia (2002). Integrated optical density (IOD), which is linearly related to the amount of DNA, was used to estimate the relative amount of DNA in individual nuclei. The IOD was measured for 200–300 interphase nuclei per slide. *Pisum sativum* cv. Kleine Rheinlaenderin was used as the calibration standard species (2C-value = 8.84 pg DNA; Greilhuber and Ebert, 1994) to convert arbitrary units to picograms of DNA. Seeds and root tips of *P. sativum* were processed in the same vials with *Z. mays* tissue throughout all experimental procedures.

Fixation, embedding, and sectioning—Caryopses of teosinte were fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% glacial acetic acid) overnight at 4°C, dehydrated in a series of ethanol and tertiary butyl alcohol, embedded in Paraplast Plus (Sherwood Medical, St. Louis, Missouri, USA), and sectioned on a rotary microtome (Autocut 2040, Reichert-Jung, Heidelberg, Germany). Three to four caryopses were collected on each day after pollination from each growing location and further analyzed in detail.

Feulgen staining and measurement of the amount of nuclear DNA in teosinte caryopsis—The amount of nuclear DNA was measured by image cytometry using the interphase-peak method (Vilhar et al., 2001; Vilhar and Dermastia, 2002) adapted for use with tissue sections (Vilhar et al., 2002; Kladnik et al., 2004). Longitudinal sections of caryopses were dewaxed in xylene, rehydrated through an ethanol series to water, hydrolyzed in 5 M HCl for 75 min at 20°C, stained with Feulgen reagent for 120 min at 20°C, washed for 45 min in six changes of SO_2-water, dehydrated in an ethanol series, then mounted in DPX (Fisons Scientific Equipment, Loughborough, UK). IOD and coordinates of the nuclei were measured on a series of grayscale images of caryopses recorded with a 40× objective. The amount of nuclear DNA was expressed in C-value units.

Toluidine blue staining—Longitudinal sections of teosinte and maize caryopses were dewaxed in xylene, rehydrated through an ethanol series to water, stained for 3 min with 0.1% toluidine blue (Paul Altmann, Berlin, Germany) in 0.1 M KH_2PO_4, washed with distilled water, quickly dehydrated and mounted in DPX. Sections stained with toluidine blue were photographed with an AxioCam MRc color digital camera (Carl Zeiss Vision, Hallbergmoos, Germany).

DAPI staining—Rehydrated sections were stained in 600 nM 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Oregon, USA), pH 7.0, for 15 min at room temperature in darkness, washed with distilled water and observed with UV excitation (excitation [ex.] 365/12 nm band pass, emission [em.] 397 nm long pass). DAPI binds preferentially to AT regions in the minor groove of double-stranded DNA and emits strong fluorescence when bound (Kubista et al., 1987). Cold-blue fluorescent nuclei were photographed with the AxioCam HRc color digital camera. DAPI-stained sections were used to count the number of enucleated cell layers in the P-C region, and statistical differences were assessed with Student's *t* test on comparable developmental stages in teosinte and maize.

Shift in cell wall autofluorescence in alkaline medium—Rehydrated sections were incubated in 0.1 M Tris buffer, pH 9.5. Control sections were incubated in distilled water only. The autofluorescence of cell walls was observed with UV excitation (ex. 365/12 nm band pass, em. 397 nm long pass) and photographed with the AxioCam MRc color digital camera. A shift in the UV-induced autofluorescence in the alkaline medium is characteristic for phenolic acids; in particular, a shift from blue to blue-green coloration specifies sinapic acid, and a shift from blue to bright blue characterizes ferulic acid and caffeic acid (Harborne, 1998).

Staining of flavonoids—Rehydrated sections were stained with a 1% methanol solution of Naturstoffreagenz A (diphenylboric acid 2-aminoethylester;

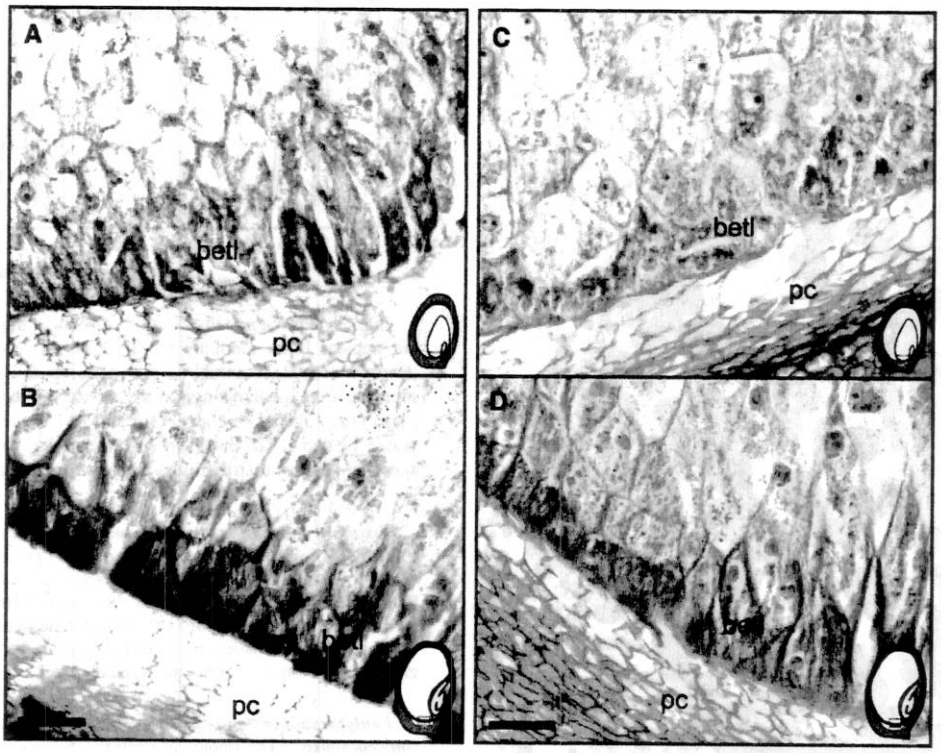


Fig. 4. Light micrographs of basal endosperm transfer layer (BETL) in toluidine-blue-stained longitudinal sections of teosinte and maize caryopses. (A) Teosinte 7 d after pollination (dap) and (B) 20 dap; (C) maize 10 dap and (D) 24 dap. Cell wall ingrowths are best seen in (B) and (D) as multiple parallel stripes. The area visible in each panel is marked by the red rectangle in the caryopsis drawing in each lower right corner. betl, basal endosperm transfer layer; pc, placento-chalazal layer. Bar = 50 μm.

Sigma-Aldrich, St. Louis, Missouri, USA), which is specific for flavonoids (Markham, 1989). Naturstoffreagenz A bound to flavonoids emits yellow-green to orange fluorescence with UV excitation (Jork et al., 1989) After 1 min of incubation, a drop of distilled water was added to the sections to prevent them from drying out due to methanol evaporation. Preparations were immediately observed with UV excitation (ex. 365/12 nm band pass, em. 397 nm long pass) and photographed with the AxioCam MRc color digital camera.

TUNEL staining—The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction is used to incorporate labeled nucleotides into DNA strand breaks with free 3′-OH ends to determine the apoptotic nature of dying cells (Gavrieli et al., 1992). The reaction was performed using an ApopTag fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, California, USA), essentially following the manufacturer's protocol. Briefly, rehydrated sections were treated with 125 μg/mL pronase E (Sigma-Aldrich) for 30 min at room temperature, incubated in a mixture of digoxigenin-labeled deoxynucleotides and TdT for 60 min at 37°C, followed by incubation with fluorescein-labeled antidigoxigenin antibodies. Slides were washed with phosphate-buffered saline (PBS) and mounted in FluoroMount (Sigma-Aldrich) with 600 nM DAPI. The fluorescein-labeled nuclei were observed with blue-light excitation (ex. 450–490 nm band pass, em. 515 nm long pass), and DAPI fluorescence of all nuclei was observed with UV excitation (ex. 365/12 nm band pass, em. 397 nm long pass) and photographed with the AxioCam HRc color digital camera.

Incw2 in situ hybridization—Antisense and sense digoxigenin-labeled zm*Incw2* (GenBank accession AF050128) RNA probes were synthesized using a DIG RNA Labeling Kit SP6/T7 (Roche Diagnostics, Mannheim, Germany) from a template maize *Incw2* cDNA clone (931 bp) amplified with PCR using forward primer TGAAGCCCTCGCACAAC and reverse primer TTGAACACCCTGAAGAACACC in pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). Probes ca. 300 bases long were generated by alkaline hydrolysis. FAA-fixed teosinte and maize caryopsis Paraplast Plus-embedded sections were placed on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany), rehydrated, treated with 125 μg/mL pronase E (Sigma-Aldrich) for 30 min at room temperature, acetylated in 0.1 M triethanolamine-HCl pH 8.0 and 0.5% acetic anhydride (both Acros Organics, Geel, Belgium), washed in PBS, dehydrated in an ethanol series and air-dried. Hybridization parameters were designed to allow pairing of nonperfect sequence matches between the two subspecies. Sections were hybridized with antisense and sense zm*Incw2* probes in a formamide-free hybridization buffer (10% dextran sulfate, 1× Denhardt's solution, 1× Na salts, 1 μg/mL tRNA) in HybChamber chambers (Genomic Solutions, Ann Arbor, Michigan, USA) for 20 h in a 58°C water bath. Posthybridization washes were two stringent 60 min washes in 0.1× SSC (sodium chloride–sodium citrate buffer) with 0.1% Triton X-100 at 58°C with gentle shaking, followed by a PBS-wash, 30 min incubation in blocking solution (5% BSA in PBS), 1 h incubation with 1:500 Anti-DIG Fab fragments conjugated with alkaline phosphatase (AP) (Roche Diagnostics), three 10 min washes in PBS + 0.1% Triton X-100, equilibration in AP-substrate buffer (0.1 M Tris-HCl pH 9.5 + 0.1 M NaCl), incubation in NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Roche Diagnostics) for 2 h and washing with distilled water. Sections were quickly dehydrated in an ethanol series and xylene and mounted in Permount (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). The purple precipitate at the sites of hybridization was photographed using a Zeiss AxioImager Z1 microscope and AxioCam HRc color digital camera.

RESULTS

Nuclear genome size—The nuclear genome size of *Zea mays* subsp. *parviglumis* estimated by interphase-peak image cytometry (Vilhar et al., 2001; Vilhar and Dermastia, 2002) and expressed as a 2C-value was 5.64 ± 0.06 pg DNA (mean ± SE, $N = 14$). The C-values of *Zea mays* subsp. *mays*, inbreds CE-777 and W22 were 5.33 ± 0.10 pg (mean ± SE, $N = 4$) and

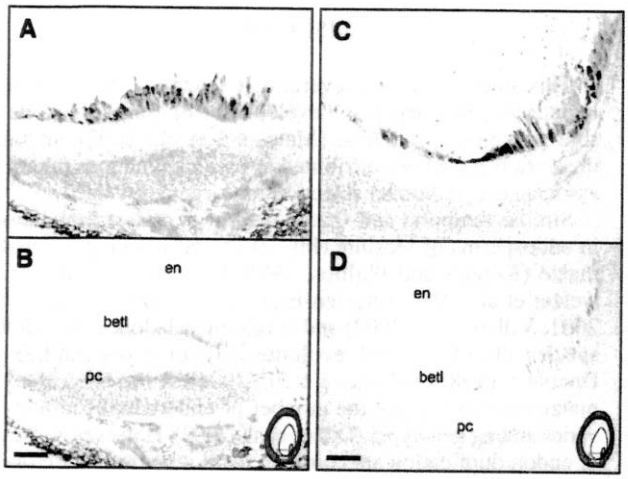


Fig. 5. Light micrographs of in situ hybridization of cell wall invertase zm*Incw2* in endosperm of (A, B) teosinte 7 d after pollination (dap) and (C, D) maize 8 dap. (A, C) Longitudinal caryopsis sections were hybridized with zm*Incw2* antisense RNA probes and the location of hybrids with cell wall invertase mRNA is visible as purple staining. (B, D) Negative controls hybridized with sense probes. The area visible in the micrographs is marked by the red rectangle in the schematic caryopsis drawings in lower right corner of (B) and (D). betl, basal endosperm transfer layer; en, endosperm; pc, placento-chalazal layer. Bar = 100 μm.

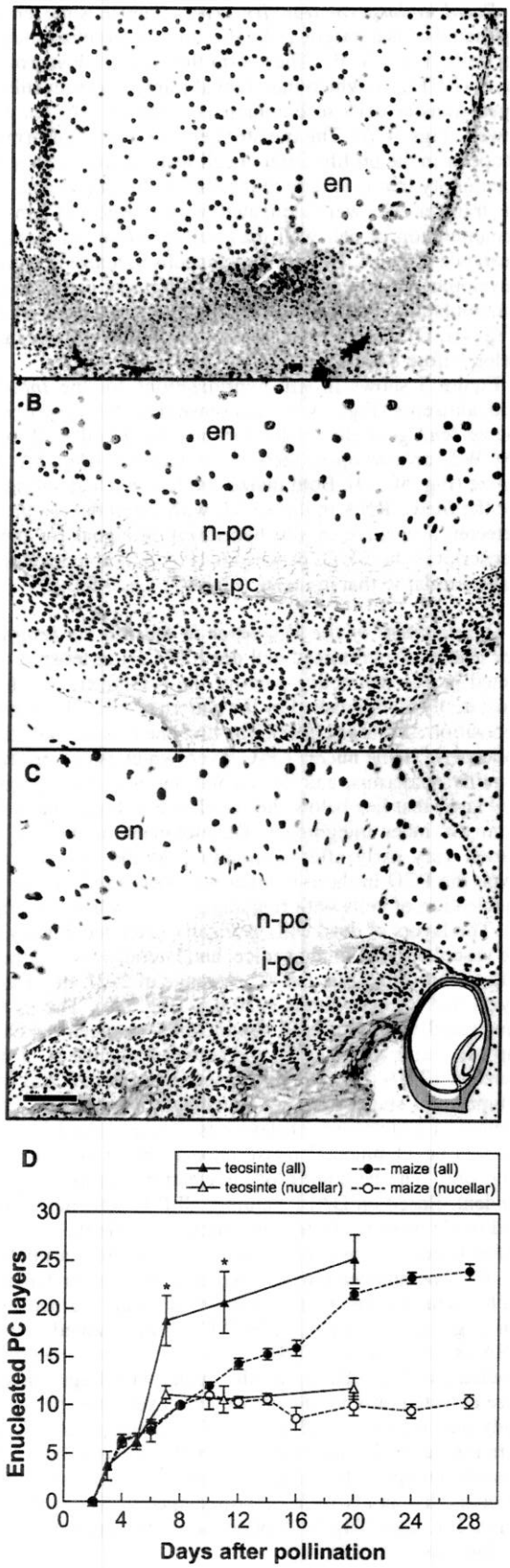
5.57 ± 0.03 pg (mean ± SE, $N = 4$), respectively. Our estimates of the teosinte C-value equates well with an earlier estimate of 5.88 ± 0.22 for its nuclear genome size (Laurie and Bennett, 1985). C-values of maize were in accordance with the median 2C-value of 5.60 pg of DNA for 74 accessions of *Z. mays* lines in the Plant DNA C-values database (Bennett and Leitch, 2005) and with an independent estimate for CE-777 of 2C = 5.43 pg DNA (Lysák and Doležel, 1998). Those data support previous observations on similarities between both subspecies in their chromosome number, gene structure, and nucleotide sequences (Doebley 1990, 2004).

DNA endoreduplication in filial endosperm and maternal pericarp nuclei—Figure 2 depicts endopolyploid nuclei in teosinte caryopsis at 11 dap. Nuclear ploidy level and size were estimated by image cytometry using the interphase peak method described previously (Vilhar et al., 2002). Because developmental progression from a mitotic to an endoreduplication phase is gradual, the endosperm had a heterogeneous population of endopolyploid nuclei (Fig. 2). The first endopolyploid nuclei with at least one endoreduplication cycle completed appeared ~5 dap, a stage at which cellularization of the syncytial endosperm is complete. Subsequently, the endosperm differentiates into the central region, where cells cease to divide, but cells in the peripheral layers are still dividing. The highest level of endoreduplication detected in teosinte endosperm was 96C (Figs. 2, 3A). Cells with higher endoreduplicated nuclei were uniformly distributed throughout the central endosperm (Fig. 2). There was a clear positive relationship between the endopolyploidy level and nuclear size in teosinte endosperm (Fig. 2). Nuclear ploidy level in the maternal tissue of pericarp in teosinte caryopsis at 11 dap was predominantly 2 and 4C (Figs. 2, 3B), while endoreduplicated nuclei with 8C DNA content were extremely rare.

Basal endosperm transfer layer—The teosinte endosperm epithelial cells adjacent to the P-C area differentiated into transfer cells (Fig. 4A, B). They form the basal endosperm transfer layer (BETL) in which the gradual transition from fully developed transfer cells to common endosperm cells may be followed (Figs. 4, 5). The transfer cells were not uniform in their shape or in the proliferation of cell wall ingrowths, a major cellular entity that is unique to the BETL. In longitudinal section, the transfer cells were elongated, with a large and non-uniform accumulation of cell wall material, giving them a striped appearance (Fig. 4A, B). The transfer cells in maize at comparable developmental stages had similar patterns of cell wall material accumulation, but they were less elongated than in teosinte (Fig. 4C, D). In both subspecies, the transfer cells began to develop before 8 dap (Fig. 4).

Figure 5 shows in situ hybridizations for the *Incw2* RNA with antisense (Fig. 5A, C) and sense (Fig. 5B, D) probes of the maize *Mn1* gene that codes for the type 2 cell wall invertase, INCW2, on caryopsis sections of teosinte (Fig. 5A, B) and maize (Fig. 5C, D). Both maize and teosinte had strong signals for the *Incw2* RNA in the BETL with the antisense probe. We detected a positive in situ hybridization signal for the *Incw2* transcript in the BETL of teosinte (Fig. 5A) at a developmental stage similar to that in maize (Fig. 5C), i.e., ~8 DAP.

Development of the P-C layer in teosinte by programmed cell death—Programmed cell death (**PCD**) in teosinte was detected in two distinctive parts of the P-C layer, which can be most easily distinguished by the pattern of phenolic compounds deposition (discussed later). The first one is just below the basal endosperm in the nucellar P-C layer, which is derived from the nucellar epidermis, and the second one is in the integumental P-C layer that lies below the nucellar P-C layer and is derived from the inner integument. Development of the P-C region started very early after pollination. At the light microscopy level, the PCD in the P-C layer was visualized by the gradual enucleation of cells with remaining intact cell walls (Fig. 6A–C). The layers of dead cells gradually extended basally toward the vascular tissue in the pedicel and laterally toward the dorsal side of caryopsis (Fig. 6A–C). A range of 2–25 enucleated cell layers had formed within 3–20 dap (Fig. 6D). The number of enucleated layers differed significantly between teosinte at 7 dap and maize at 8–12 dap, and between teosinte at 11 dap and maize at 12–16 dap. The difference in the earlier and later developmental stages was not statistically significant. The final number of enucleated cell layers is similar in both subspecies, but this developmental phase appears to be slower in maize (Fig. 6D). The beginning of enucleation was fertilization-dependent. Based on DAPI staining, all P-C cells in the unfertilized ovule were nucleated (not shown), while the same region in fertilized caryopses had layers of cells without nuclei (Fig. 6A–C). The enucleation was preceded by the condensation of nuclei, which were small, round and strongly stained by Feulgen (Fig. 7A). The nuclei in the P-C layer contained fragmented DNA as detected by TUNEL reaction at the very beginning of enucleation (Fig. 7B). Later, the nuclei with fragmented DNA were detected only in one or two cell layers, just below the already enucleated cells (Fig. 7C). The development of the teosinte P-C layer was accompanied by the deposition of different phenolic compounds in the remaining cell walls (Fig. 8A, B). Whereas flavonoids were deposited specifically in the integumental P-C layer (Fig. 8A), phenolic acids were present in both subdomains (Fig. 8B).



DISCUSSION

This study describes several cellular traits and mechanisms underlying the caryopsis development of Balsas teosinte, the sole progenitor of maize (Matsuoka et al., 2002). In maize, these traits have been attributed to various functions potentially associated with human selection for larger seed size.

Similar temporal and spatial pattern of endoreduplication as in endosperms of teosinte (Fig. 2) has been seen previously in maize (Kowles and Phillips, 1985; Kowles et al., 1997; Schweizer et al., 1995; Engelen-Eigles et al., 2000; Larkins et al., 2001; Vilhar et al., 2002) and sorghum (Kladnik et al., 2006), a species closely related evolutionarily to maize (White and Doebley, 1998; Swigoňová et al., 2004). It has been shown in maize endosperm that the number of endoreduplication cycles varies among genotypes (Dilkes et al., 2002). However, 4–5 cycles of endoreduplication are common to all inbreds, and Schweizer et al. (1995) showed that the level of endoreduplication as a function of days after pollination remains the same from one yearly crop to another. In addition, in our 8-yr study of the development of maize W22 caryopsis, we did not observe any significant changes in endoreduplication in relation to different growing seasons (Vilhar et al., 2002; Kladnik et al., 2004, 2005; Chourey et al., 2006; LeClere et al., 2007). We also followed the development of a nonvouchered teosinte caryopsis during several growing seasons, and in one season the development of a caryopsis of accession 21812 (data not shown). It is noteworthy that they were not distinctive from the development described in this work. It might be possible that the endoreduplication varies to some degree in other teosinte lines, but it is not very likely that it would be generally affected by the teosinte genotype. Collectively, these findings support the idea that endoreduplication resulting in extremely high levels of DNA is a well-conserved feature of endosperm development in cereals (Kowles and Phillips, 1985; Caro et al., 2007; Nguyen et al., 2007). However, the main difference between the endoreduplication in endosperm of teosinte or maize was in the distribution of the cells with highly endoreduplicated nuclei. While in teosinte such cells were distributed throughout the central endosperm (Fig. 2), they were limited to the upper part of the central endosperm in maize (Vilhar et al., 2002). In teosinte (Fig. 2), maize (Vilhar et al., 2002), and sorghum (Kladnik et al., 2006), there was a positive relationship between

Fig. 6. Epifluorescence micrographs of progression of cell death in the placento-chalazal (P-C) layer of teosinte. (A) Early developmental stage ~3 d after pollination (dap); arrow points to the first enucleated cells. (B) Intermediate stage at 7 dap. (C) Mature stage ~20 dap. Longitudinal sections of teosinte caryopses were stained with DAPI, photographed, and presented as grayscale negatives for enhanced contrast; nuclei are seen as black dots, cell walls are visible by their autofluorescence. The area of enucleated cells in the P-C layer is outlined with white dashed lines in (C); the area visible in the micrographs is marked by a dotted rectangle in the caryopsis drawing in lower right. (D) Temporal changes in the number of cell layers with enucleated cells (seen as empty in A–C) in the P-C region in teosinte and maize P-C layer. Open symbols: number of enucleated cell layers only in the nucellar P-C; solid symbols: number of enucleated cell layers in both nucellar and integumental subdomains. Data for maize was adapted from Kladnik et al. (2004). Error bars represent SD, number of enucleated cell layers was measured in 3–4 caryopses for each developmental stage, * $P < 0.05$ (t test). en, endosperm; i-pc, integumental P-C layer; n-pc, nucellar P-C layer. Bar = 100 μm.

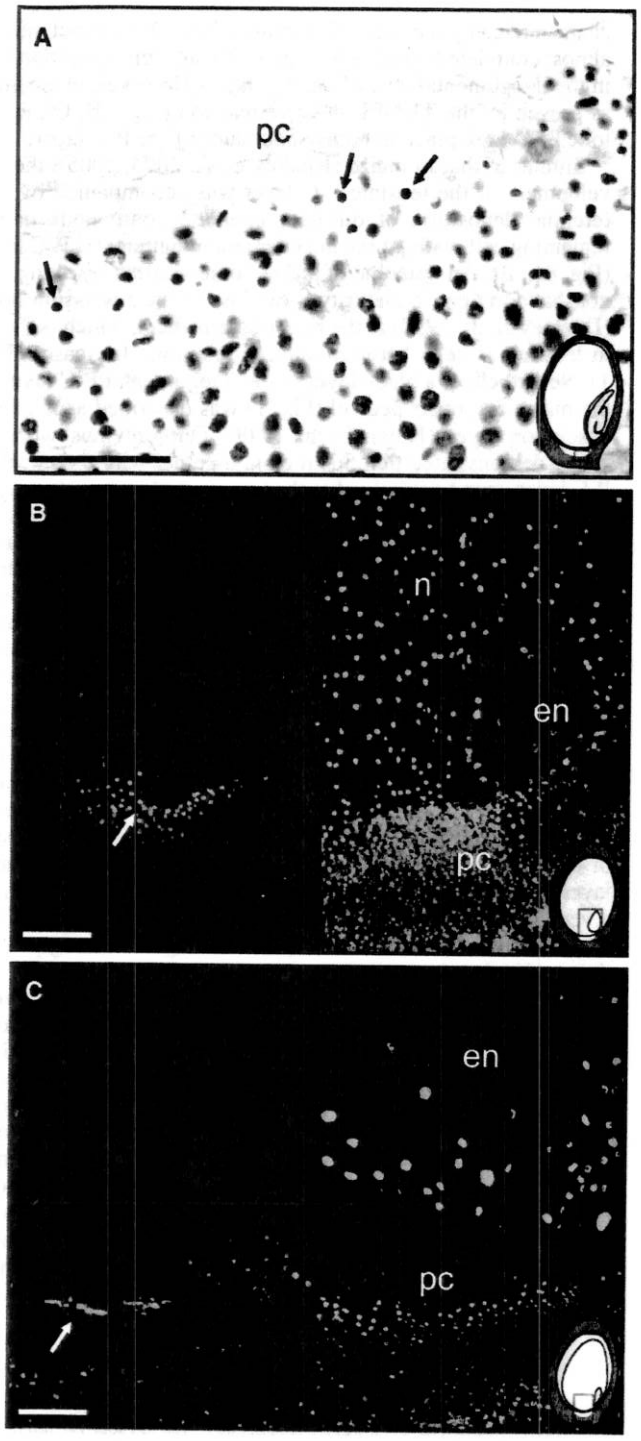


Fig. 7. Light micrographs of nuclei condensation in the placento-chalazal (P-C) layer of teosinte and epifluorescence micrographs of nuclear DNA fragmentation in teosinte caryopsis. (A) Feulgen-stained section of the teosinte P-C layer ~20 d after pollination (dap); condensed nuclei in cells immediately below the already dead cells are indicated by arrows. (B, C) TUNEL staining of nuclei indicating fragmented DNA is visible as bright green fluorescence (arrows) in the still nucleated layers of the P-C region of teosinte caryopsis in left panels in (B) ~3 dap and (C) 7 dap; right panels show the same sections that were control-stained with DAPI that stains all nuclei. The area in each image is marked by the red rectangle in the schematic caryopsis drawing in lower right. en, endosperm; n, nucellus; pc, P-C layer. Bar in A = 50 μm, bars in B, C = 100 μm.

endoreduplication and the size of nuclei. While there is often also a correlation between cell size and nuclear ploidy, endoreduplication is not necessarily coupled with cell size or high levels of gene expression, although it could be associated with processes that are coincident with these events (Nguyen, 2007). Such a positive correlation between endoreduplication and the cell size has been demonstrated in maize and sorghum endosperms (Vilhar et al., 2002; Kladnik et al., 2006). In sorghum, this relationship is associated with starch deposition (Kladnik et al., 2006). It might be possible, therefore, that the specific distribution of cells with highly endoreduplicated nuclei in the upper part of the maize caryopsis is related to more efficient starch deposition as a consequence of domestication.

The basal endosperm cells of maize confined to cells adjacent to the P-C differentiate into the transfer cells to form BETL, which is demonstrated to be critical for normal seed development. One of the functions most often postulated for BETL is nutrient acquisition from the maternal postphloem region in the pedicel to the symplastically isolated, developing maize seed (reviewed in Thompson et al., 2001). BETL is thus spatially and temporally the first filial cell layer that the photosynthates enter from the mother plant. Cell wall invertase is crucial to normal seed development in maize. The lack of the *Mn1*-encoded cell wall invertase, INCW2, in the maize BETL is the causal basis of the *miniature1* (*mn1*) seed phenotype (Cheng et al., 1996), which is associated with reduced cell size and cell number in developing endosperm (Vilhar et al., 2002) and a loss of nearly 70% of the seed mass relative to the wild type. Furthermore, it is significant to note that the *Mn1*-encoded INCW2 controls the flux of sucrose entering a developing seed as evidenced by a positive gene-dosage correlation between the levels of INCW2 activity in the developing endosperm and in the seed mass (Cheng et al., 1996). The *mn1* seed mutant is, however, nonlethal, presumably because of the residual low level (~1% of the wild type) of cell wall invertase activity (Miller and Chourey, 1992) encoded by a nonallelic locus, *Incw1* (Chourey et al., 2006). The expression of *Incw2* RNA in the BETL of teosinte endosperm (Fig. 5) indicates a similar role of INCW2 in teosinte and likely a common mechanism for sugar uptake in both subspecies. As measured by quantitative real-time PCR, the transcript abundance of the teosinte *Incw1* gene (GenBank accession AF050129) was 10 times lower than that of *Incw2*, and similar to maize, the transcript abundance decreased as the caryopsis matured (data not shown).

Development of the apoplastic space in the P-C region, which is directly in contact with the endosperm, had several features of PCD (Figs. 6–8), and as in maize (Kladnik et al., 2004), its initiation was fertilization-dependent. The most prominent feature was the gradual enucleation of P-C cell layers as shown with light microscopy. It was finished by ~20 dap (Fig. 6). On the other hand, the enucleation was extended in maize (Fig. 6D). This observation agrees with the faster maturation of the teosinte caryopsis in comparison with maize and its disarticulation in less than 1 month. Using transmission electron microscopy and TUNEL staining, we have shown in maize that the most prominent feature of PCD in the P-C layer is its duality (Kladnik et al., 2004, 2005). While in the nucellar P-C layer, PCD was

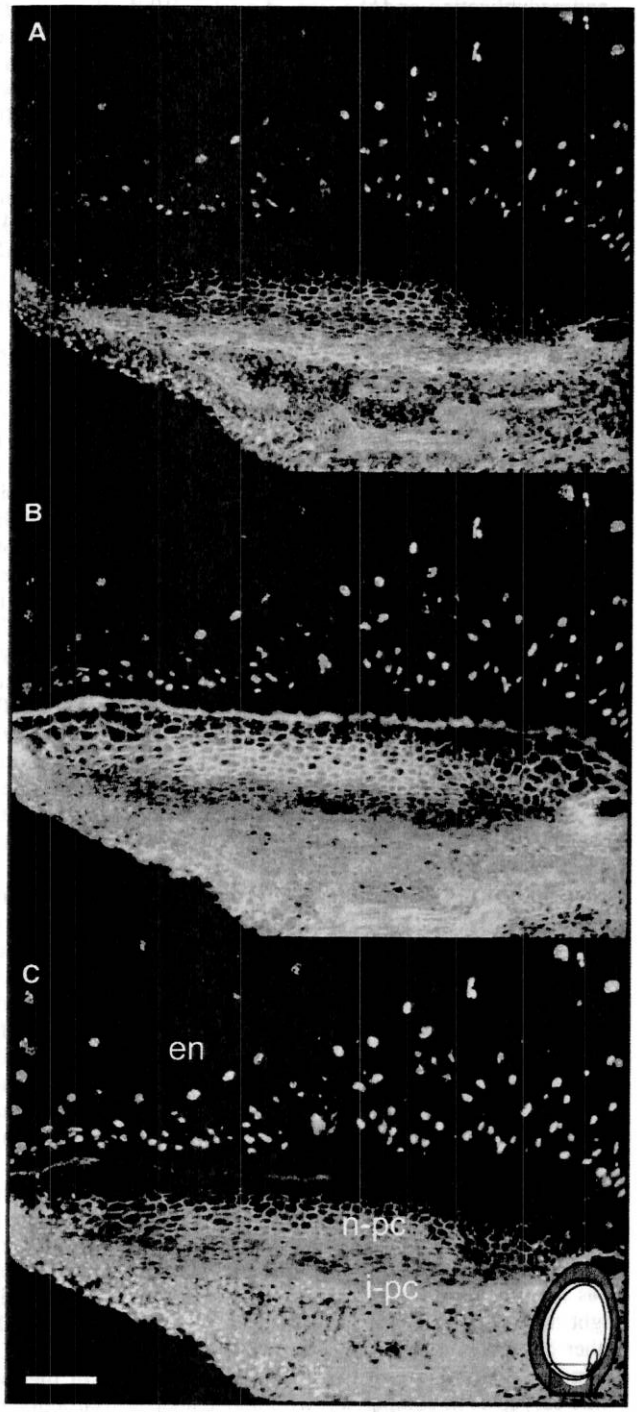


Fig. 8. Epifluorescence micrographs of phenolic compounds in the placento-chalazal (P-C) layer of teosinte 7 d after pollination. (A) Distribution of flavonoids in the integumental subdomain of the P-C layer. (B) Phenolic acids in both subdomains of the P-C layer. (C) Control section with autofluorescence in tissue incubated in distilled water. All micrographs were photographed using UV excitation, see Materials and Methods for filter set details. The area visible in the micrographs is marked by the red rectangle in the schematic caryopsis drawing in (C). en, endosperm; i-pc, integumental P-C layer; n-pc, nucellar P-C layer. Bar = 100 μm.

characterized by the autolytic rupture of the vacuole and then an almost complete loss of cell content, it was more apoptotic-like in the integumental part of the P-C layer. However, in teosinte, as proven by the TUNEL-positive reaction (Fig. 7B, C), apoptotic PCD took place in both subdomains of the P-C layer.

Similar to that in maize (Kladnik et al., 2004, 2005), the development of the teosinte P-C layer was accompanied by differential deposition of different phenolic compounds in the remaining cell walls of the nucellar and integumental P-C layer (Fig. 8A, B). An outcome of maize domestication resulting in a nonshattering ear at maturity is the loss of the abscission layer (Doebley et al., 1990) at the base of the pedicel, which is found in teosinte as cells between adjacent cupulate fruitcases (Fig. 1). Nevertheless, a black layer in the integumental P-C layer of the maize caryopsis pedicel (Fig. 1) was described as a brown abscission layer (Kiesselbach, 1949). Our previous work in maize demonstrated that this region, together with the nucellar P-C layer, may be involved in the transport of water, photosynthates and nutrients from the mother plant to the filial tissues. In this system, the remaining cell walls would act as water-transporting cells in the xylem, i.e., the tracheary elements (Kladnik et al., 2004). Tracheary elements undergo a rapid PCD prior to becoming functionally mature (for a review, see McCann, 1997) and their development includes the deposition of phenolic compounds to strengthen the cell walls and protect them against decay and pathogens. Our hypothesis of a similarly developed transport system in the maize P-C layer was supported by the occurrence of the PCD of the P-C layer cells and the deposition of phenolic acids and flavonoids in their cell walls (Kladnik et al., 2004, 2005; LeClere et al., 2007). The appearance of a separate black layer in maize is thus merely the result of differences in the phenolic compounds in the integumental layer from those in the nucellar P-C layer. The confirmation of a very similar development of the teosinte P-C layer including PCD and the accumulation of phenolics agrees strongly with the previously proposed assumption.

In conclusion, our results indicate that the essential developmental cellular processes in the caryopsis evolved before maize domestication and do not contribute to the striking changes in the structure of the maize caryopsis phenotype compared with that in Balsas teosinte. Notably, only the specific distribution of large cells with very large endopolyploid nuclei in the upper central endosperm of maize, which is not observed in teosinte, might contribute to more effective storage of starch. On the basis of these results that show conservation of the entire cellular program seen previously in maize, we suggest that these features evolved independently of human selection pressure and domestication in the developing teosinte caryopsis.

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